EXHIBIT H

Impairment of Neocortical Long-Term Potentiation in Mice Deficient of Endothelial Nitric Oxide Synthase

SVEN HAUL,[1] AXEL GÖDECKE,[2] JÜRGEN SCHRADER,[2,3] HELMUT L. HAAS,[1,3] AND HEIKO J. LUHMANN[1,3]
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Haul, Sven, Axel Gödecke, Jürgen Schrader, Helmut L. Haas, and Heiko J. Luhmann. Impairment of neocortical long-term potentiation in mice deficient of endothelial nitric oxide synthase. *J. Neurophysiol.* 81: 494–497, 1999. The role of the possible retrograde messenger nitric oxide (NO) in the induction of long-term potentiation (LTP) was studied in supragranular layers of somatosensory cortical slices obtained from adult mice. High-frequency stimulation produced a slowly rising, long-lasting (50 min) and significant ($P < 0.001$) increase in the extracellular synaptic response by 23%. The induction of LTP was independent from activation of *N*-methyl-D-aspartate (NMDA) receptors, but prevented by bath application of N^G-nitro-L-arginine methyl ester (L-NAME), indicating that one or several of the different NO synthases (NOS) produced NO within the postsynaptic neuron. No LTP could be induced in knockout mice lacking the endothelial NOS (eNOS) isoform. These data suggest that eNOS is involved in an NMDA receptor–independent form of LTP in the rodent cerebral cortex.

INTRODUCTION

Since the pioneering work of Bliss and Lømo (1973), long-term potentiation (LTP) has been extensively studied in various brain regions as a model for learning and memory (for review, see Bliss and Collingridge 1993). The molecular processes leading to a prolonged increase in synaptic efficacy have been attributed to presynaptic and/or postsynaptic mechanisms (for review, Madison and Schuman 1991). A favorite hypothesis postulates the existence of a retrograde messenger that travels from the postsynaptic site back to the presynapse to cause changes in the release of excitatory transmitters (Arancio et al. 1996). Arachidonic acid, nitric oxide (NO), and carbon monoxide are candidate molecules to fulfill the criteria of a retrograde messenger (O'Dell et al. 1991; Zhuo et al. 1993). Inhibition of NO synthase (NOS) has been shown to suppress LTP, both under in vivo (Iga et al. 1993; Mizutani et al. 1993), and in vitro (Bon et al. 1992; Haley et al. 1992) conditions (but see also Bannerman et al. 1994). In addition, the involvement of the different NOS isoforms, neuronal (type I, nNOS), inducible (type II, iNOS), and endothelial (type III, eNOS), in hippocampal LTP has been recently studied in more detail in mutant mice deficient of nNOS ($nNOS^-$) (O'Dell et al. 1994). These data indicate that eNOS, rather than nNOS, is the primary source of NO in the postsynaptic neuron during LTP. This hypothesis has been confirmed by two different groups using recombinant adenovirus vectors containing a truncated eNOS (Kantor et al. 1996) and mutant mice deficient of eNOS ($eNOS^-$) (Wilson et al. 1997). In contrast, Son et al. (1996) demonstrated that hippocampal LTP was normal in $eNOS^-$ mice, but reduced in CA1 stratum radiatum of doubly mutant mice ($nNOS^-/eNOS^-$). We were interested in the extent that eNOS contributes to neocortical synaptic plasticity and studied the expression of LTP in somatosensory cortical slices of $eNOS^-$ mice.

METHODS

Twenty-six control mice and seven eNOS-deficient mice with an age of 8–12 wk were used for this study. eNOS knockout mice were constructed by deletion of exons 24825 of the eNOS gene, which resulted in the disruption of the essential NADPH binding site. Homozygous animals have no eNOS activity and develop hypertension (for details of construction and characterization of this mouse, see Gödecke et al. 1998). For all experiments, control and knockout mice were siblings derived from F1 heterozygous intercrosses.

The methods for preparing and maintaining neocortical slices in vitro were similar to those described previously (Luhmann et al. 1995). In brief, 400-μm-thick coronal slices of the primary somatosensory cortex were cut on a vibratome in ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose with a pH of 7.4 when saturated with 95% O_2-5% CO_2. Slices were transferred to an incubation-storage chamber or to an interface-type recording chamber and kept at 31–32°C. Slices were allowed to recover for at least 1.5 h before recording began. Extracellular recordings were performed with 2–5 MΩ electrodes filled with ACSF. Extracellular field potential responses in layers II/III to orthodromic synaptic stimulation of the underlying layer IV were elicited at intervals of 60 s. For obtaining input-output curves, the stimulus duration was increased in steps of 20 μs from 40 to 300 μs. For the LTP experiments, the pulse duration was fixed at 200 μs, and the stimulus strength was adjusted to an intensity that evoked a submaximal field response of at least 1 mV in amplitude. Only slices with stable responses showing variations in amplitude <5% during baseline recording were used for analysis. After 15 min baseline recording, 200-ms-long 100-Hz stimulus trains were delivered every 5 s to the afferent pathway for 10 min. This high-frequency stimulation (HFS) reliably produced LTP by 20–30% (Aroniadou and Teyler 1992). After this 10-min high-frequency stimulation, synaptic responses were recorded every 60 s for a period of 60 min. Data were digitized on-line and analyzed off-line using the TIDA software program (HEKA, Lambrecht, Germany). The field amplitude was measured between the negative- and positive-going peak of the orthodromic response. For pharmacological analyses, the selective *N*-methyl-D-aspartate (NMDA) receptor antagonist DL-amino-phosphonovaleric acid (APV, Sigma, Basel) was applied locally in a concentration of 120–200 μM (dissolved in ACSF) with a broken micropipette placed on the slice surface near the recording site. In experiments designed to investigate the effect of NOS inhibition, slices were incubated for >1.5 h in ACSF containing 200 μM N^G-nitro-L-arginine methyl ester (L-NAME; Sigma) and transferred to

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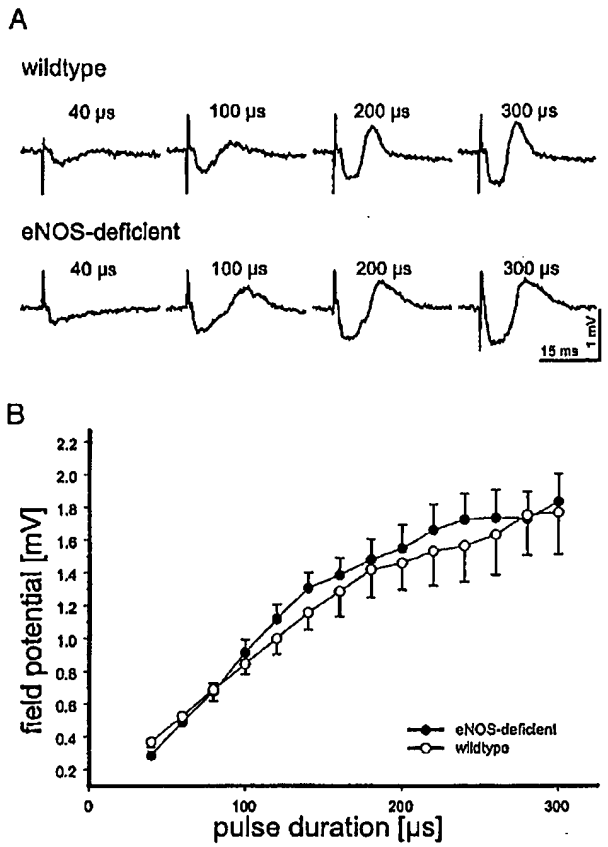

FIG. 1. Input-output relationship of stimulus-evoked field potential responses in wildtype and eNOS$^-$ mice. *A*: responses to electrical stimuli of various duration (40, 100, 200, and 300 μs) in wildtype (*top row*) and eNOS$^-$ mice (*bottom row*). *B*: absolute field potential amplitudes to stimuli ranging from 40 to 300 μs in wildtype (○, n = 7 slices) and eNOS$^-$ mice (●, n = 8). Data are expressed as means ± SE. No significant difference between both groups could be obtained over the whole range of stimulus durations. eNOS, endothelial NOS.

the recording chamber containing the same bathing solution. For statistical analyses, five subsequent responses before and 50 min after HFS were compared by the Student's t-test. If not otherwise noted, values throughout this report are expressed as means ± SE.

RESULTS

Neocortical slices prepared from wild type and eNOS$^-$ mice were analyzed in basal excitatory synaptic transmission by recording field potential responses to electrical stimulation of the afferents with constant intensity and various durations (Fig. 1). In both experimental groups, short stimulus pulses (40–80 μs) elicited a small orthodromic response (Fig. 1*A*). An increase in stimulus duration evoked a larger field response that reached a maximum at a stimulus duration of 240–300 μs (Fig. 1*B*). The input-output curves for both groups were very similar, and no statistically significant difference could be detected between wildtype and eNOS$^-$ mice. In wildtype mice, HFS for 10 min produced a gradual increase in the orthodromic field potential response, which after 50 min reached a value of 122.7 ± 3.6% (mean ± SE; n = 11, $P < 0.001$) of the baseline

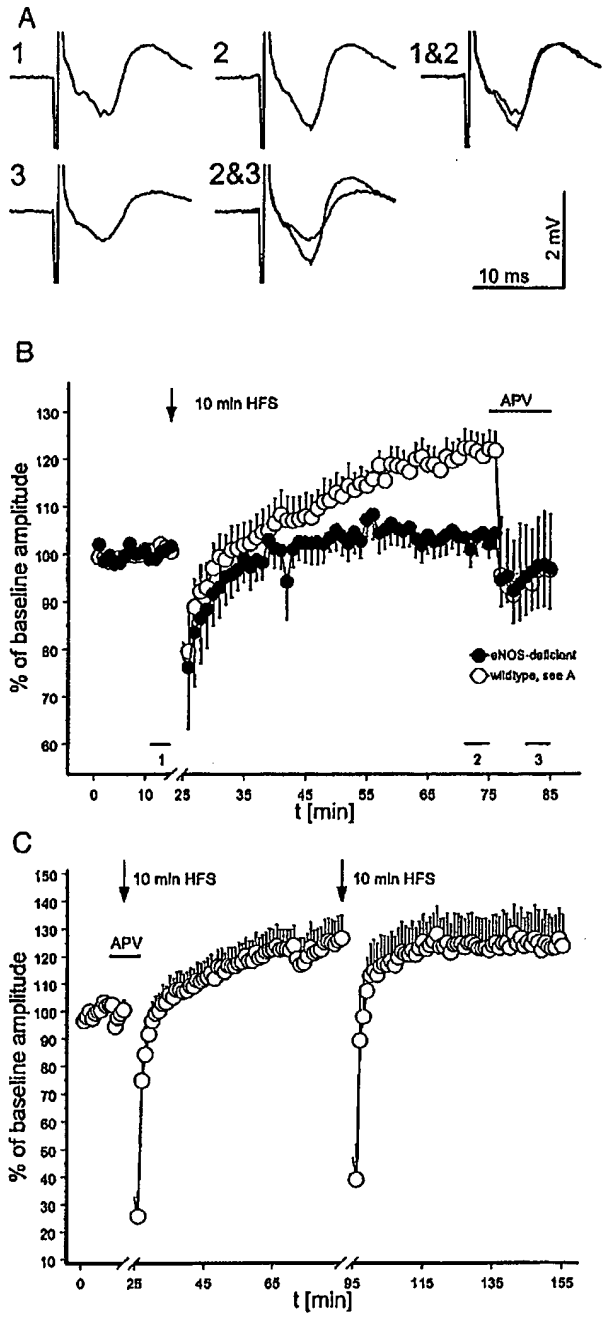

FIG. 2. Neocortical long-term potentiation (LTP) in adult wildtype (○) and eNOS$^-$ (●) mice. *A*: characteristic field potential responses in layers II/III to orthodromic synaptic stimulation before (*1*) and 50 min after high-frequency stimulation (HFS; *2*) recorded in somatosensory cortex of a wildtype mouse. Application of the selective *N*-methyl-d-aspartate (NMDA) antagonist DL-amino-phosphonovaleric acid (APV) causes a blockade of the potentiated response (*3*). Time points *1–3* are also indicated in *B*. *B*: expression of LTP in wildtype mice (○, n = 11) and lack of LTP in eNOS$^-$ mice (●, n = 10). Local application of APV causes a blockade of LTP in wildtype mice and small decrease of synaptic responses in eNOS$^-$ mice. *C*: induction of LTP in wildtype mice (○, n = 10) is not prevented by local application of APV. A 2nd HFS does not induce a further potentiation.

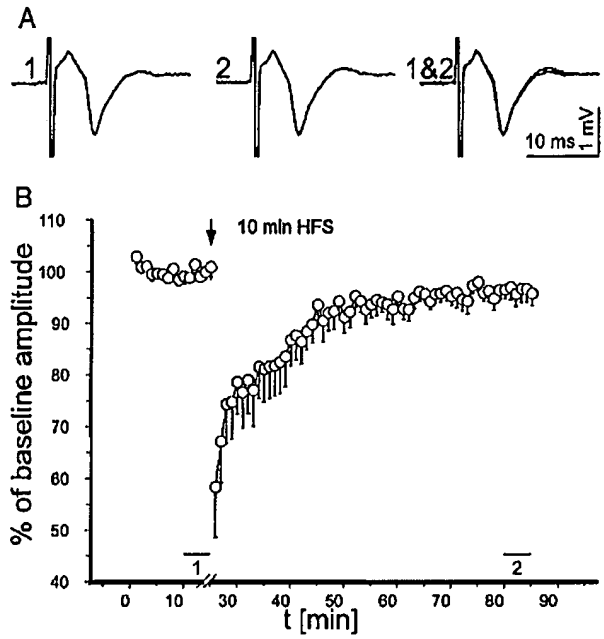

FIG. 3. Blockade of neocortical LTP by NOS inhibition with 200 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME). *A*: single field potential responses before (*1*) and 50 min after HFS (*2*). Note identical amplitude of both responses when traces are superimposed (*1&2*). *B*: average field potential amplitudes recorded in neocortical slices exposed to L-NAME (n = 10).

control (Fig. 2, *A1* and *A2* and Fig. 2*B*, ○). Local application of the selective NMDA antagonist APV to the potentiated slice caused in wildtype mice a significant ($P < 0.05$) reduction in the field potential amplitude to 94.8 ± 10.6% (Fig. 2*A3* and APV in Fig. 2*B*, 10 min after APV application), indicating that the potentiated response was mediated by an NMDA receptor–dependent synaptic component. In contrast, slices from eNOS$^-$ mice did not reveal any significant increase in the field potential response (Fig. 2*B*, ●). Fifty minutes after HFS stimulation, the average field amplitude in eNOS$^-$ mice amounted to only 102.1 ± 2.8% (n = 10). In agreement with our observations on wildtype mice, application of the NMDA antagonist APV to slices prepared from eNOS$^-$ animals produced an insignificant reduction in the field response to 97 ± 8.2% (APV in Fig. 2*B*).

The role of NMDA receptors in the induction of LTP was studied in parietal cortical slices from wildtype mice. Application of APV during the HFS did not prevent the induction of LTP (Fig. 2*C*). After termination of the HFS and wash out of APV, the postsynaptic response gradually increased significantly ($P < 0.02$) to 125.6 ± 8.8% (n = 10). A second HFS 60 min after termination of the first HFS did not induce any further potentiation in the field potential response (124 ± 11.9%, Fig. 2*C*). These data suggest that the stimulus protocol induced an NMDA receptor–independent form of LTP. In another control experiment, we tested the effects of an NOS inhibitor on the induction of LTP in neocortical slices obtained from wildtype mice. Bath application of the NOS inhibitor L-NAME prevented the induction of LTP in wildtype mice (n = 10, Fig. 3), indicating that the stimulus protocol produced a potentiation that was completely mediated by the effects of NO.

DISCUSSION

The major conclusions from our study are that *1*) mouse neocortical slices reveal a slow-onset LTP, which in its induction is NMDA receptor independent and in its maintenance NMDA receptor dependent; *2*) that this form of LTP can be prevented by blockade of NO synthase in the postsynaptic neuron; and *3*) that this NO synthase is predominantly mediated by the eNOS isoform. Because the induction of NO-mediated LTP is strongly stimulus dependent (Chetkovich et al. 1993; Wilson et al. 1997), the lack of LTP in eNOS$^-$ mice may have been attributed to an impairment in basal excitatory synaptic transmission. However, the input-output response curves did not differ between both experimental groups, suggesting that our protocol produced the same orthodromic synaptic stimulation. The gradual increase in the response amplitude in wildtype mice is in good agreement with earlier observations obtained in adult rodent hippocampal (Bashir et al. 1993) and neocortical slices (Aroniadou and Teyler 1992; Kirkwood et al. 1993). Furthermore, a slow-onset form of LTP not requiring the activation of NMDA receptors for its induction has been previously reported in area CA1 on HFS (Grover and Teyler 1990) and after application of metabotropic glutamate receptor agonist (Bashir et al. 1993). Whereas the former type of LTP was suppressed by a voltage-dependent calcium antagonist (Grover and Teyler 1990), the latter form of LTP was prevented by an antagonist acting at the metabotropic glutamate receptor (Bashir et al. 1993). These data suggest that the HFS protocol used in the present report induced an NMDA receptor–independent form of LTP, which may depend on calcium entry via voltage-gated calcium channels or inositol trisphosphate–mediated calcium release from intracellular stores after activation of metabotropic glutamate receptors (Wilsch et al. 1998). The discrepancy between ours and the recent observations of Hensch et al. (1998) in mouse visual cortical slices demonstrating the prerequisite of NMDA receptor for the induction of a fast-onset LTP may be explained by the different stimulus protocols (see Grover and Teyler 1990) or different ages of the animals (see Crair and Malenka 1995).

Our observation on the pronounced APV sensitivity of the potentiated response is surprising. A potentiation of an NMDA receptor–mediated response with a latency of 4–7 ms has been occasionally observed in the rat motor cortex (Aroniadou and Keller 1995), but the magnitude of this APV-sensitive potentiated field potential component was considerably smaller. The relatively low magnesium concentration of 1 mM used in our experiments certainly contributes to a stronger potentiation of an NMDA receptor–mediated component. In agreement with previous reports on hippocampal (O'Dell et al. 1991) and neocortical (Nowicky and Bindman 1993) slices, demonstrating a blockade of LTP in the presence of NOS inhibitors, we also observed in wildtype mice a prevention of LTP by the NOS inhibitor L-NAME, indicating that the stimulus protocol produced a potentiation that was completely mediated by the effects of NO. These data are also compatible with immunocytochemical studies illustrating moderately high levels of NOS in the supragranular levels of the rodent cerebral cortex (Bredt et al. 1990). Taken together, these data suggest that NO plays an important role in the induction of LTP in the cerebral cortex of adult mice.

The experimental analysis of neocortical slices obtained

from mice deficient of eNOS allowed the investigation of the question whether this isoform is the critical synthase in mediating this form of neocortical LTP. Slices from eNOS$^-$ mice showed the usual posttetanic depression but did not reveal any significant increase in the field response, suggesting that eNOS, rather than nNOS, is the dominant synthase of NO production in the postsynaptic neuron during LTP. This result corresponds well to previous in vitro data in nNOS$^-$ mice, which expressed normal hippocampal LTP (O'Dell et al. 1994), and recent observations on eNOS$^-$ mice, which showed no hippocampal LTP to weak stimuli (Wilson et al. 1997). Furthermore, Kantor et al. (1996) reported that rat hippocampal slices treated with an adenovirus vector containing a truncated eNOS revealed no LTP in stratum radiatum. These data strongly suggest that the eNOS isoform plays an important role in hippocampal synaptic plasticity. However, Son et al. (1996) have shown that LTP in stratum radiatum was most profoundly reduced in doubly mutant mice (nNOS$^-$/eNOS$^-$), but normal in nNOS$^-$ animals and eNOS$^-$ mice.

Our observations suggest that eNOS is also involved in neocortical LTP, but that the magnitude of potentiation is smaller in the cerebral cortex than in the hippocampus. Immunocytochemical studies have shown that staining for eNOS is much weaker in the rodent neocortex as compared with the hippocampus (Dinerman et al. 1994). This difference in the localization of eNOS may be one of the reasons why neocortical LTP shares many features with hippocampal LTP but usually reaches a much smaller amplitude (Kirkwood et al. 1993).

This work was supported by Deutsche Forschungsgemeinschaft Grants Lu 375/3–1 and 3–2 to H. J. Luhmann, DFG Grant "Synaptic Interactions" to H. L. Haas, and a grant from the Fritz-Thyssen-Stiftung Köln to J. Schrader.

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Received 15 July 1997; accepted in final form 3 October 1998.

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